

## Research article

**A novel approach in the treatment of neuroendocrine gastrointestinal tumors: Additive antiproliferative effects of interferon- $\gamma$  and meta-iodobenzylguanidine**Michael Höpfner<sup>1</sup>, Andreas P Sutter<sup>1</sup>, Alexander Huether<sup>1</sup>, Gudrun Ahnert-Hilger<sup>2</sup> and Hans Scherübl<sup>\*1</sup>

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**Abstract**

**Background:** Therapeutic options to effectively inhibit growth and spread of neuroendocrine gastrointestinal tumors are still limited. As both meta-iodobenzylguanidine (MIBG) and interferon- $\gamma$  (IFN $\gamma$ ) cause antineoplastic effects in neuroendocrine gastrointestinal tumor cells, we investigated the antiproliferative effects of the combination of IFN $\gamma$  and non-radiolabeled MIBG in neuroendocrine gut STC-I and pancreatic carcinoid BON tumor cells.

**Methods and results:** IFN $\gamma$  receptors were expressed in both models. IFN $\gamma$  dose- and time-dependently inhibited the growth of both STC-I and of BON tumor cells with IC<sub>50</sub>-values of  $95 \pm 15$  U/ml and  $135 \pm 10$  U/ml, respectively. Above 10 U/ml IFN $\gamma$  induced apoptosis-specific caspase-3 activity in a time-dependent manner in either cell line and caused a dose-dependent arrest in the S-phase of the cell cycle. Furthermore, IFN $\gamma$  induced cytotoxic effects in NE tumor cells.

The NE tumor-targeted drug MIBG is selectively taken up via norepinephrine transporters, thereby specifically inhibiting growth in NE tumor cells. Intriguingly, IFN $\gamma$  treatment induced an upregulation of norepinephrine transporter expression in neuroendocrine tumors cells, as determined by semi-quantitative RT-PCR. Co-application of sub-IC<sub>50</sub> concentrations of IFN $\gamma$  and MIBG led to additive growth inhibitory effects, which were mainly due to increased cytotoxicity and S-phase arrest of the cell cycle.

**Conclusion:** Our data show that IFN $\gamma$  exerts antiproliferative effects on neuroendocrine gastrointestinal tumor cells by inducing cell cycle arrest, apoptosis and cytotoxicity. The combination of IFN $\gamma$  with the NE tumor-targeted agent MIBG leads to effective growth control at reduced doses of either drug. Thus, the administration of IFN $\gamma$  alone and more so, in combination with MIBG, is a promising novel approach in the treatment of neuroendocrine gastrointestinal tumors.

## Background

Gastrointestinal neuroendocrine (NE) tumors represent a rare and rather heterogeneous tumor entity. Almost half of the metastatic NE gastrointestinal tumors release excessive amounts of biogenic amines and/or neuropeptides thereby causing a characteristic hypersecretion syndrome. The often bizarre clinical symptoms are generally well controlled by somatostatin analogues or interferon- $\alpha$  (IFN $\alpha$ ) [1,2]. However, further growth and spread is generally not prevented on the long by either biotherapy or conventional chemotherapy. Thus, innovative treatment strategies should be developed that are both effective and well tolerated.

A promising approach for novel treatment of NE gastrointestinal tumor disease may be the modulation of the IFN $\gamma$ -receptor system. The immunomodulatory lymphokine IFN $\gamma$  modulates biological responses by binding to its cognate receptor thereby activating members of a transcription factor family termed STAT (signal transducers and activators of transcription) [3]. IFN $\gamma$ -induced STAT signaling and STAT-associated induction of interferon regulatory factors (IRFs) have been shown to exert key regulatory function for proliferation, differentiation and survival [4]. The underlying mechanisms of IFN $\gamma$ -induced growth inhibition show a broad spectrum, including the induction of cell cycle arrest, apoptosis and cytotoxicity [5,6].

Although interferons are known to evoke growth regulatory effects in many tissues, the effectiveness of IFN-induced growth inhibition varies between the various cell types. Thus, growth control observed in a given cell type does not allow to predict similar effects of IFN $\gamma$  on tissues of other origin [7].

IFN $\gamma$ -induced growth inhibition in NE gastrointestinal tumors has not yet been evaluated in great detail. Here we examine the antineoplastic potency of IFN $\gamma$  alone or in combination with non-radiolabeled MIBG in NE gastrointestinal tumor cells of the gut and pancreas.

In its radiolabeled form the norepinephrine analog MIBG is an established diagnostic and therapeutic agent in neuroendocrine tumors expressing plasma membrane bound norepinephrine transporters [8-10]. Recently, we and others could show that even its non-radiolabeled form, the so-called "cold MIBG" can effectively inhibit the growth of neuroendocrine tumor cells [11,12]. Since IFN $\gamma$  has been reported to upregulate the MIBG-transporting norepinephrine transporter in neuroblastoma cells [13], it was intriguing to evaluate, whether this also held true for NE gastrointestinal tumors cells and whether such an upregulation could boost the antineoplastic potency of cold MIBG in NE tumor cells.

## Methods

### Cell lines

Human pancreatic carcinoid BON cells [14,15] were maintained in a 1:1 mixture of DMEM, and F12K medium containing 10% FCS (Biochrom, Berlin, Germany) and 1% L-glutamine. The murine intestinal neuroendocrine tumor cell line STC-1, which was a gift from Dr. D. Hanahan (University of California, San Francisco, CA), was maintained in DMEM supplemented with 15% horse serum (Biochrom, Berlin, Germany), 2.5% FCS (Biochrom) and 1% L-glutamine [16]. The neuroendocrine-differentiated human colorectal cancer cell line Colo 320 DM [17] was maintained in standard RPMI 1640 medium, complemented with 10% FCS. All cell lines were kept at 37°C in a humidified atmosphere (5% CO<sub>2</sub>).

### Drugs

IFN $\gamma$  and non-radiolabeled meta-iodobenzylguanidine (MIBG) were purchased from Sigma (München, Germany). Stock solutions were prepared in water or DMSO, respectively, and stored at -20°C. The drugs were diluted in fresh media before each experiment. In all experiments, the final DMSO concentration was <0.5%. To evaluate the effects of IFN $\gamma$  and/or MIBG, cells were incubated with either control medium or medium containing rising concentrations of the respective drugs. Media were changed daily to ensure constant drug concentrations in the incubation medium.

### RT-PCR

Semi-quantitative analysis of mRNA expression of the human plasma membrane bound norepinephrine transporter (hNET) and the housekeeping gene  $\beta$ -actin was carried out by RT-PCR with the number of cycles at which the band intensity increased linearly with the amount of mRNA used [18].

Total RNA was extracted from cultured cell lines with RNAClean following the recommendation of the manufacturer (Hybaid, London, UK). Reverse transcription and PCR reactions were carried out as described elsewhere in detail [19]. To eliminate any possible contamination with genomic DNA, RNAs were treated with 1 U DNase I (Gibco, Karlsruhe, Germany) per  $\mu$ g RNA for 15 min at room temperature. Purified RNA was reverse transcribed into cDNA using oligo-dT-primers and the SuperScript Preamplification-Kit (Gibco). PCR reactions were carried out in a total volume of 50  $\mu$ l containing 400 nM of each primer, 200  $\mu$ M of each dNTP (Pharmacia, Uppsala, Sweden), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris and 1 U Taq-Polymerase (Pharmacia). PCR was performed in a Peltier thermocycler (PTC-200, MJ Research, Watertown, MA) with primers and at the conditions indicated in table 1.

**Table 1: Primer sequences and PCR conditions used to evaluate the expression of the transcripts indicated**

Genes	Primers (5'-3')	Product size (bp)	Denaturing temperature and time (s)	Annealing temperature and time (s)	Extension temperature and time (s)	number of cycles
IFN $\gamma$ R	F:ACgCagAAggAAgATgATTgTgACg R:TCTATTggAgTCagATggCTgCCC	590	94°C (30)	63°C (90)	72°C (90)	32
hNET	F:CTggTggTgAAggAgCgCAACggC R:ATgTgATgAATCCCgCTgCTCTCg	241	94°C (60)	63°C (60)	72°C (60)	32
$\beta$ -actin	F:ATCATgTTTgAgACCTTCAACAC R:TCTgCgCAAgTTAgTTTTgTC	822	94°C (60)	63°C (60)	72°C (60)	32

All templates were initially denaturated for 5 min at 95°C and the amplification was followed by a final extension temperature of 72°C for 7 min.

### Measurement of growth inhibition

Changes in cell number of BON and STC-1 cells were determined by crystal violet staining after incubation with rising drug concentrations (IFN $\gamma$ : 0–500 U/ml; MIBG: 0–100  $\mu$ M) for up to 5 days. Measurements were performed as described [20]. Cells were washed with PBS and fixed with 1% glutaraldehyde. After another washing step, cells were stained with 0.1% crystal violet. The unbound dye was removed by washing. Crystal violet that had absorbed onto the cells was solubilized with 0.2% Triton X-100. Then light extinction was analyzed at 570 nm using an ELISA reader.

### Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelev and Christensen, as described previously [21]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson, Heidelberg, Germany).

### Caspase-3 activity assay

To determine apoptosis the activity of caspase-3, a key enzyme in the apoptotic cascade was measured. Preparation of cell lysates and determination of caspase-3 activity was performed as described previously [22]. The activity of caspase-3 was calculated from the cleavage of the fluorogenic substrate DEVD-AMC (Calbiochem-Novabiochem, Bad Soden, Germany). Cell lysates were incubated with substrate solution (caspase-3 substrate AC-DEVD-AMC 20  $\mu$ g/ml, HEPES 20 mM, glycerol 10%, DTT 2 mM, pH 7.5) for 1 h at 37°C, and the cleavage of DEVD-AMC was measured fluorometrically with a VersaFluor fluorometer (excitation: 360 nm emission: 460 nm) from Biorad, Munich, Germany.

### Determination of cytotoxicity

Cells were seeded at a density of 5,000 cells/well into 96-well microtiter plates and incubated with IFN $\gamma$  (0–500 U/ml) or MIBG (0–50  $\mu$ M) or the combination of both. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured after 48 h of incubation by using the colorimetric kit from Roche as described elsewhere [20,23]. Background release obtained by measuring LDH release of untreated cells was subtracted. Maximum release of LDH was obtained by adding 100  $\mu$ l of 2% Triton X-100 to untreated cells. For determinations, 100  $\mu$ l of each sample were transferred to a 96-well microtiter plate; 100  $\mu$ l of LDH assay reagent were added to each well and incubated for 30 min at room temperature in the dark. The absorbance of samples was then measured at 490 nm. No significant LDH release was detected in the tissue culture medium alone nor in drug-containing incubation medium.

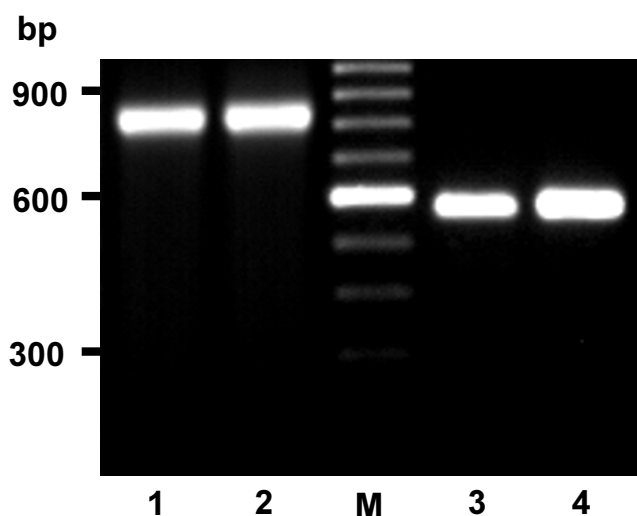
### Statistical analysis

The antineoplastic effects of the various substances and vehicles were compared by the unpaired, two-tailed Mann-Whitney U-test. The unpaired student t-test was used for cell cycle analysis. P values were considered to be significant at <0.05. If not stated otherwise, all functional experiments were performed in quadruplicate.

### Results

#### Expression of IFN $\gamma$ receptors in NE gastrointestinal tumor cells

To monitor IFN $\gamma$  receptors (IFN $\gamma$ R) in human pancreatic carcinoid BON cells, mRNA expression of IFN $\gamma$ R was evaluated by RT-PCR. Using specific primers directed against the  $\alpha$ -chain of the human IFN $\gamma$ R [24], we detected a robust expression of PCR-amplificates of the expected size (Fig. 1). The neuroendocrine-differentiated colorectal carcinoma cell line Colo320 DM served as a positive control. IFN $\gamma$ R expression in murine intestinal NE STC-1 tumor cells has already been demonstrated by cDNA array analysis [11].



**Figure 1**  
**Expression of IFN $\gamma$  receptors (IFN $\gamma$ R) in neuroendocrine gastrointestinal tumor cells.** mRNA expression of IFN $\gamma$ R was evaluated in human pancreatic carcinoid BON tumor cells (lane 3). Neuroendocrine differentiated colorectal carcinoma cells (Colo320 DM) served as positive control for IFN $\gamma$ R (lane 4).  $\beta$ -actin was used as loading control (lane 1: BON, lane 2: Colo320 DM). PCR products were visualized by gel electrophoresis on an ethidium bromide-stained agarose gel. M: 100 bp DNA ladder.

#### Growth inhibition by IFN $\gamma$

Changes in cell number induced by IFN $\gamma$  were studied by performing crystal violet assays. IFN $\gamma$  (0–500 U/ml) time- and dose-dependently inhibited the growth of both BON and STC-1 cells. After 5 days of incubation a decrease in cell number of up to 90% was observed in BON cells (Fig. 2a). In slower growing STC-1 cells a decrease of up to 75% was observed after 5 days of incubation (Fig. 2b). The IC<sub>50</sub> values of IFN $\gamma$ , determined after 72 h of incubation, amounted to  $135 \pm 10$  U/ml in BON cells and  $95 \pm 15$  U/ml in STC-1 cells.

#### Additive antiproliferative effects of IFN $\gamma$ and MIBG

Recently, we showed that non-radiolabeled MIBG specifically inhibited the growth of norepinephrine transporter (NET)-expressing STC-1 tumor cells (IC<sub>50</sub>  $\sim 8$   $\mu$ M) and that MIBG-treatment increased the expression of IFN $\gamma$  receptors. Vice versa, IFN $\gamma$  also enhanced MIBG-uptake [13]. Hence, we additionally evaluated antiproliferative effects of a combination treatment of NE gastrointestinal tumor cells with IFN $\gamma$  and MIBG.

In STC-1 cells sub-IC<sub>50</sub> concentrations of IFN $\gamma$  (10–100 U/ml) and MIBG (1–5  $\mu$ M) produced impressive additive growth inhibitory effects (Fig. 3a). The combination of

100 U/ml IFN $\gamma$  with 5  $\mu$ M MIBG decreased the proliferation of STC-1 cells by 95%. Such an antiproliferative effect was reached by MIBG alone only with concentrations of at least 25  $\mu$ M.

Using RT-PCR we also demonstrated the expression of plasma membrane bound NET in BON cells which had not yet been investigated with regard to NET expression (Fig. 4a). Proliferation assays revealed that MIBG (1–100  $\mu$ M) dose- and time-dependently inhibited the growth of BON cells by up to  $68 \pm 3.4\%$ . The IC<sub>50</sub> value of MIBG, determined after 48 h of incubation, amounted to  $47.4 \pm 2.1$   $\mu$ M being distinctly higher than in STC-1 cells. Pre-treating BON cells with IFN $\gamma$  (10–500 U/ml) influenced the expression of MIBG-transporting NET. Semiquantitative RT-PCR revealed an IFN $\gamma$ -induced increase of NET expression in BON cells by up to 200% after 48 h (Fig. 4b). However, upregulation of NET expression did not result in an overadditive antiproliferative effect of IFN $\gamma$  (10–100 U/ml) plus MIBG (10–20  $\mu$ M). Like in STC-1 cells, additive antiproliferative effects of IFN $\gamma$  and MIBG were observed in BON cells (Fig. 3b). The combination of 100 U/ml IFN $\gamma$  with 10  $\mu$ M MIBG produced antiproliferative effects that were comparable to those achieved by 50  $\mu$ M MIBG on its own.

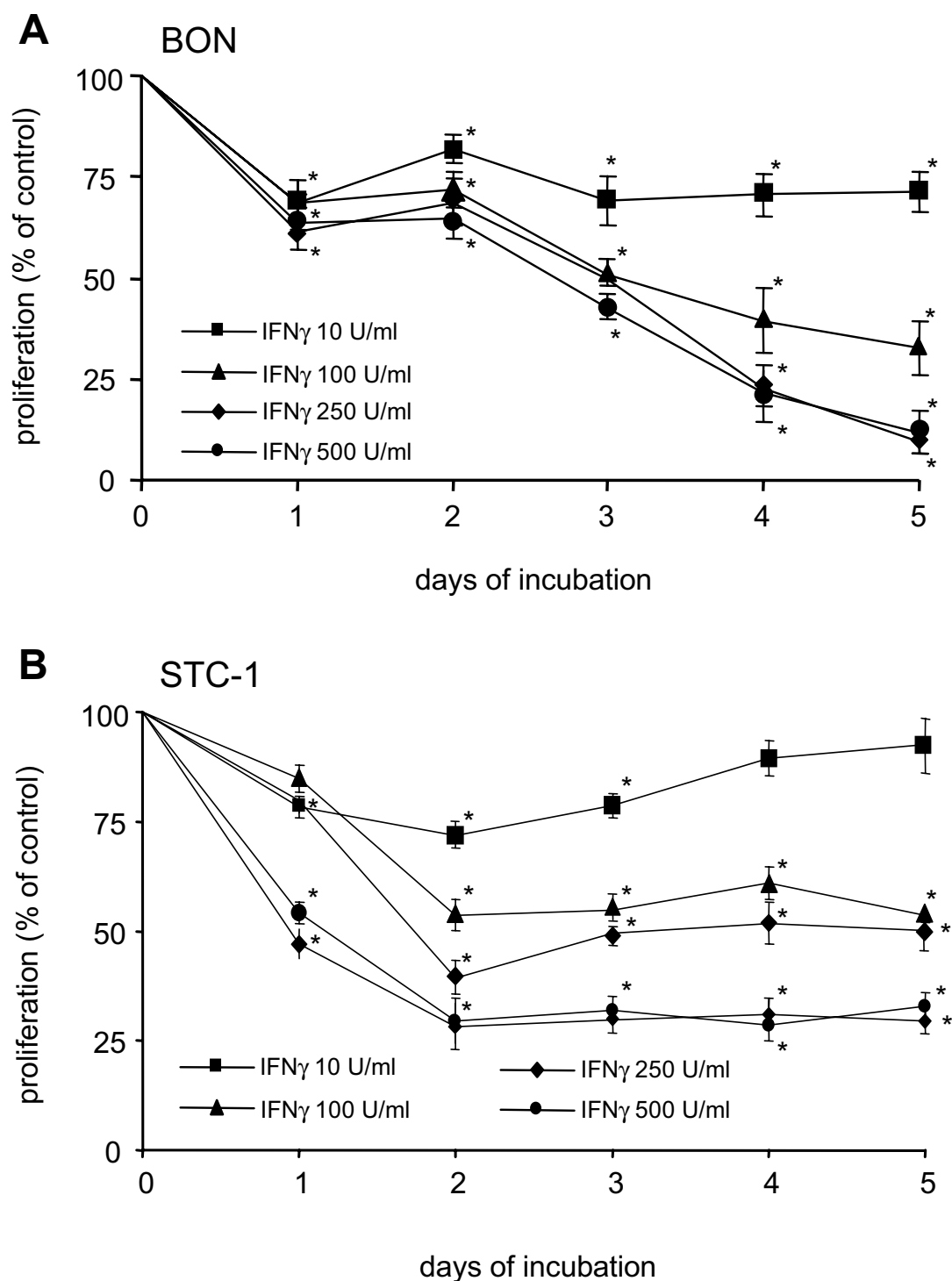
#### IFN $\gamma$ and cell cycle regulation

Flow cytometric analysis revealed that challenging BON cells for 48 h with rising concentrations of IFN $\gamma$  (0–500 U/ml) dose-dependently arrested the cells in the S-phase of the cell cycle, thereby decreasing the proportion of cells in the G<sub>2</sub>/M-phase. S-phase arrest was significant for 250–500 U/ml IFN $\gamma$  (Fig. 5). The effect of MIBG treatment on the cell cycle of BON cells was also evaluated. In contrast to slow-growing STC-1 cells, not displaying cell cycle arresting effects in response to MIBG-treatment [13], BON cells showed a significant and dose-dependent arrest in the G<sub>0</sub>/G<sub>1</sub>-phase of the cell cycle after treatment with MIBG (10–50  $\mu$ M). Accordingly, the proportion of cells in the G<sub>2</sub>/M-phase decreased.

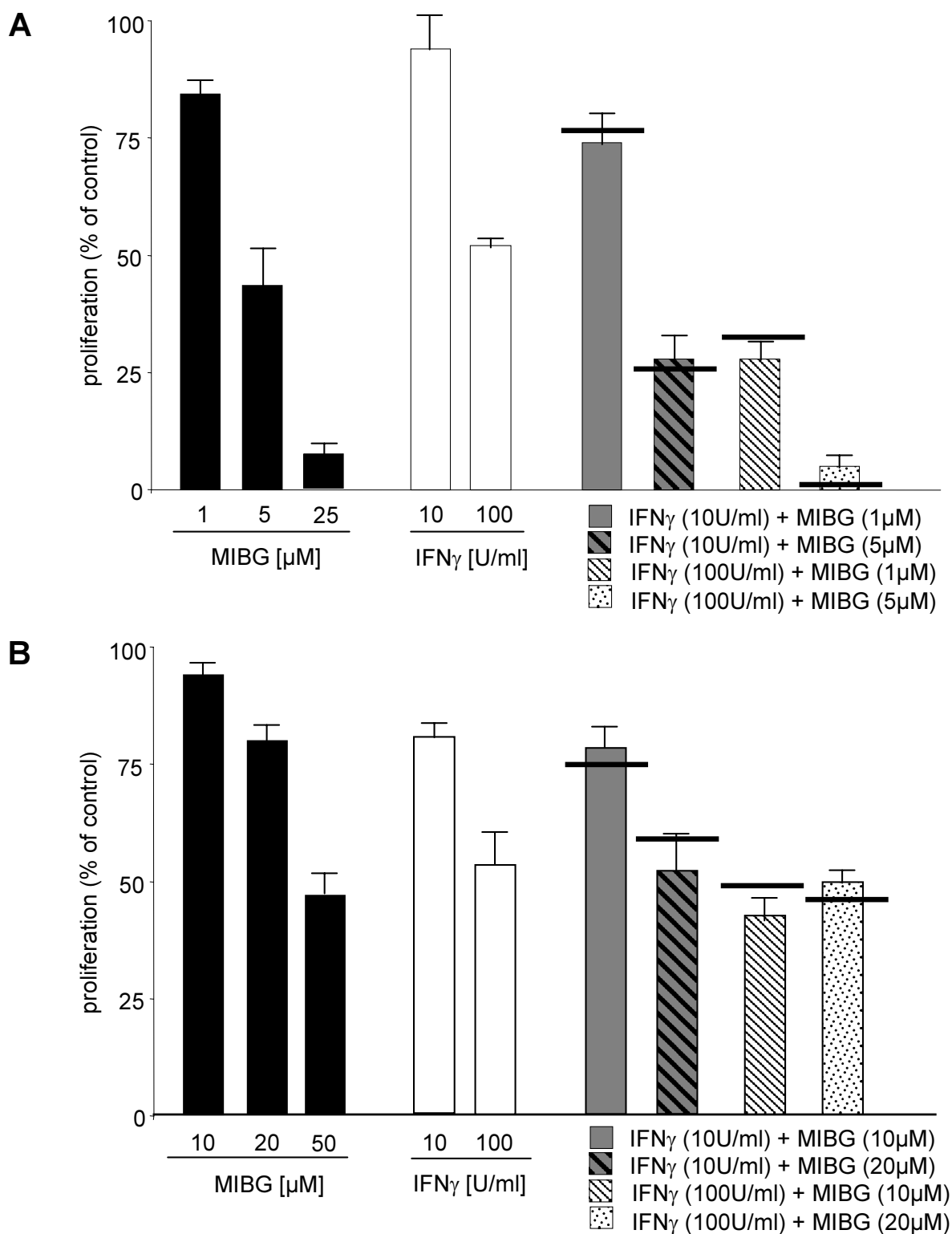
Co-application of IFN $\gamma$  (10–100 U/ml) and MIBG (50  $\mu$ M) resulted in an abrogation of the MIBG-induced G<sub>0</sub>/G<sub>1</sub>-phase arrest, while the proportion of cells in the S-phase significantly increased as compared to MIBG-treatment alone. Interestingly, the S-phase arrest induced by combination treatment with 100 U/ml IFN $\gamma$  and 50  $\mu$ M MIBG was as strong as the one achieved by 500 U/ml IFN $\gamma$  alone (Fig. 5).

#### Proapoptotic effects of IFN $\gamma$

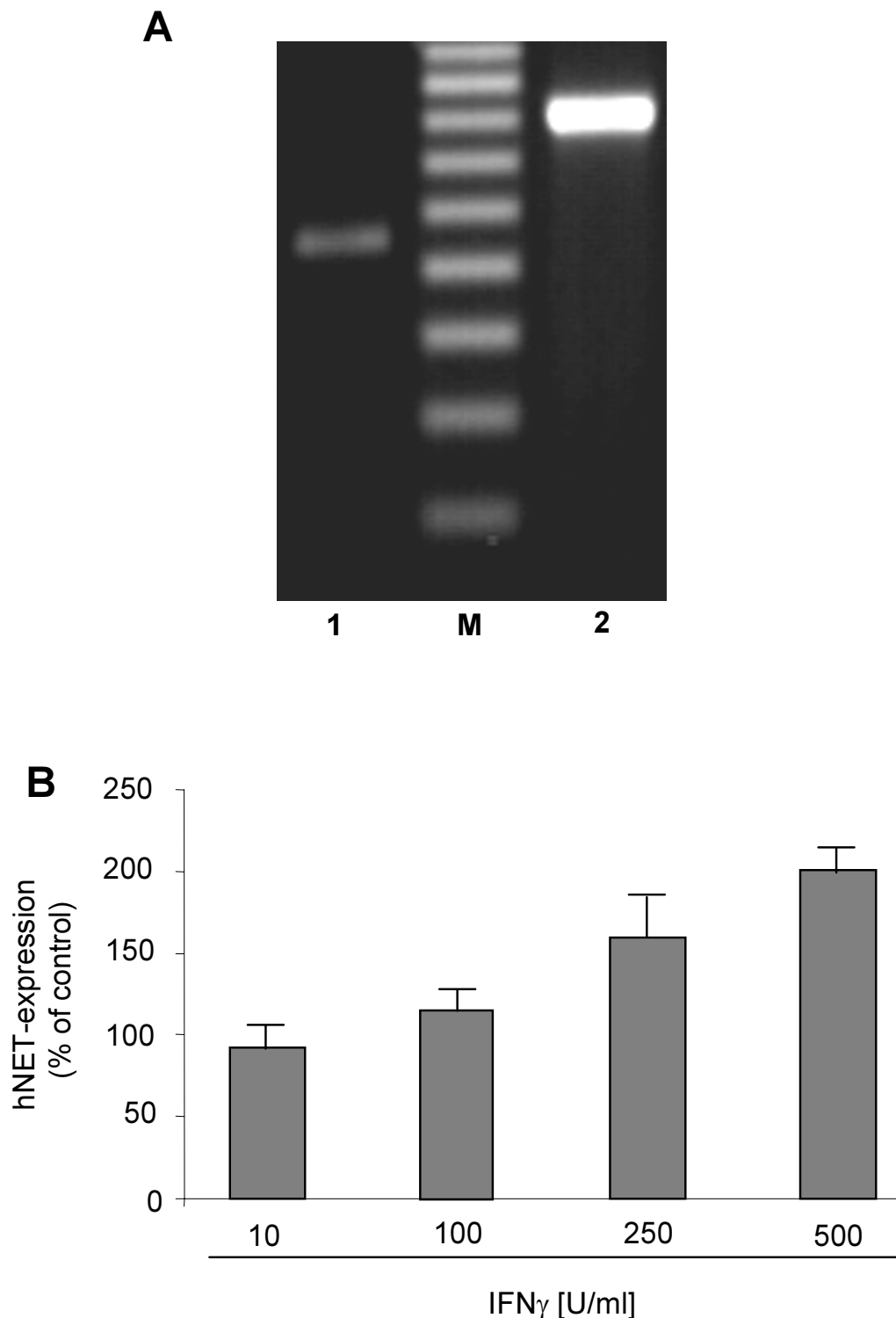
To check whether an induction of programmed cell death contributed to the antineoplastic effects of IFN $\gamma$ , we investigated IFN $\gamma$ -induced activation of caspase-3, a key enzyme in the apoptotic signaling pathway. Above 10 U/

**Figure 2**

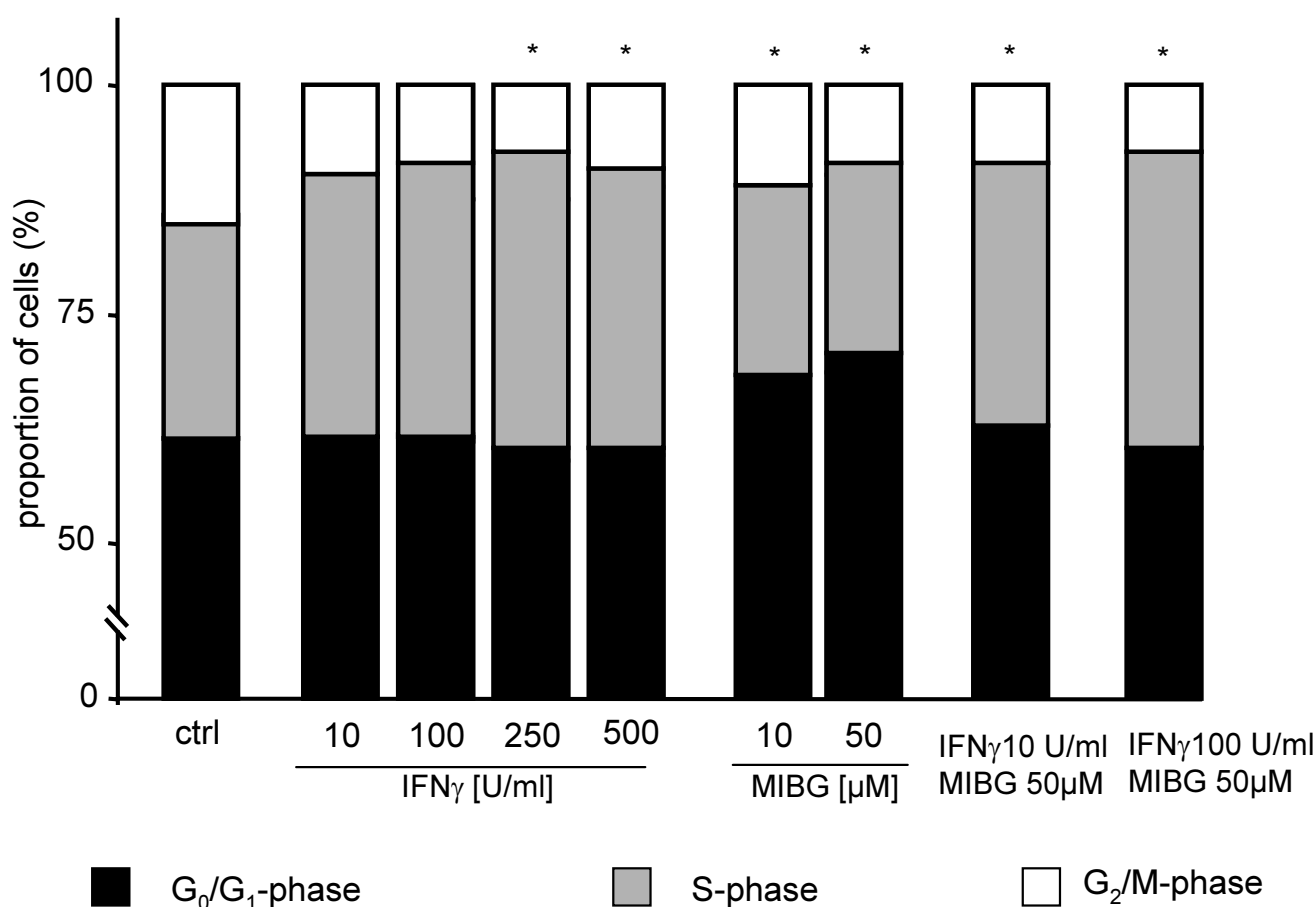
**IFN $\gamma$ -induced growth inhibition** Rising concentrations of IFN $\gamma$  for 5 days led to a time- and dose-dependent growth inhibition of neuroendocrine gastrointestinal tumor cells, as measured by crystal violet staining. The growth of BON tumor cells was inhibited with an IC<sub>50</sub> value of  $135 \pm 10$  U/ml (**A**). STC-1 cells displayed an IC<sub>50</sub> value of about 100 U/ml ( $95 \pm 15$  U/ml) (**B**). Data are given as percentage of untreated controls (means  $\pm$  SEM of 4 experiments). \*, statistical significance ( $p < 0.05$ ) compared to untreated controls.

**Figure 3**

**Antiproliferative effects of combination treatment with IFN $\gamma$  and MIBG** A combination treatment with sub-IC<sub>50</sub> concentrations of IFN $\gamma$  and MIBG for 72 h led to additive growth inhibitory effects in STC-I (**A**) and BON cells (**B**). Black bars indicate the values of a calculated additive growth inhibition. Data are given as percentage of untreated controls (means  $\pm$  SEM of 3 experiments).

**Figure 4**

**mRNA expression of human norepinephrine transporters (hNET)** mRNA expression of norepinephrine transporters was evaluated in BON cells. **A:** Transcripts specific for hNET were detected by RT-PCR (lane 1).  $\beta$ -actin was used as loading control (lane 2). PCR products were visualized by gel electrophoresis on an ethidium bromide-stained agarose gel. M: 100 bp DNA ladder. **B:** Up-regulation of hNET expression by IFN $\gamma$ . Semi-quantitative RT-PCR revealed a dose-dependent induction of hNET expression in BON cells by IFN $\gamma$  after 48 h. Data are given as means  $\pm$  SEM of 2 experiments.

**Figure 5**

**Induction of cell cycle arrest by IFN $\gamma$  and MIBG** After 48 h of incubation IFN $\gamma$  dose-dependently induced an arrest in the S-phase of the cell cycle of BON cells. Accordingly, the proportion of cells in the G<sub>2</sub>/M-phase decreased. Differences in the proportion of cells in the respective phase of cell cycle versus control were significant from 250 to 500 U/ml IFN $\gamma$  ( $p < 0.05$ ). Incubation of BON cells with MIBG for 48 h induced a dose-dependent arrest in the G<sub>0</sub>/G<sub>1</sub>-phase, while the proportion of cells in the G<sub>2</sub>/M-phase decreased. Differences in the proportion of cells in the respective phase of cell cycle versus control were significant at 10 and 50  $\mu$ M MIBG ( $p < 0.05$ ). Combination treatment of MIBG and IFN $\gamma$  abolished MIBG-induced G<sub>0</sub>/G<sub>1</sub>-phase arrest. Means of 4 independent experiments are shown.

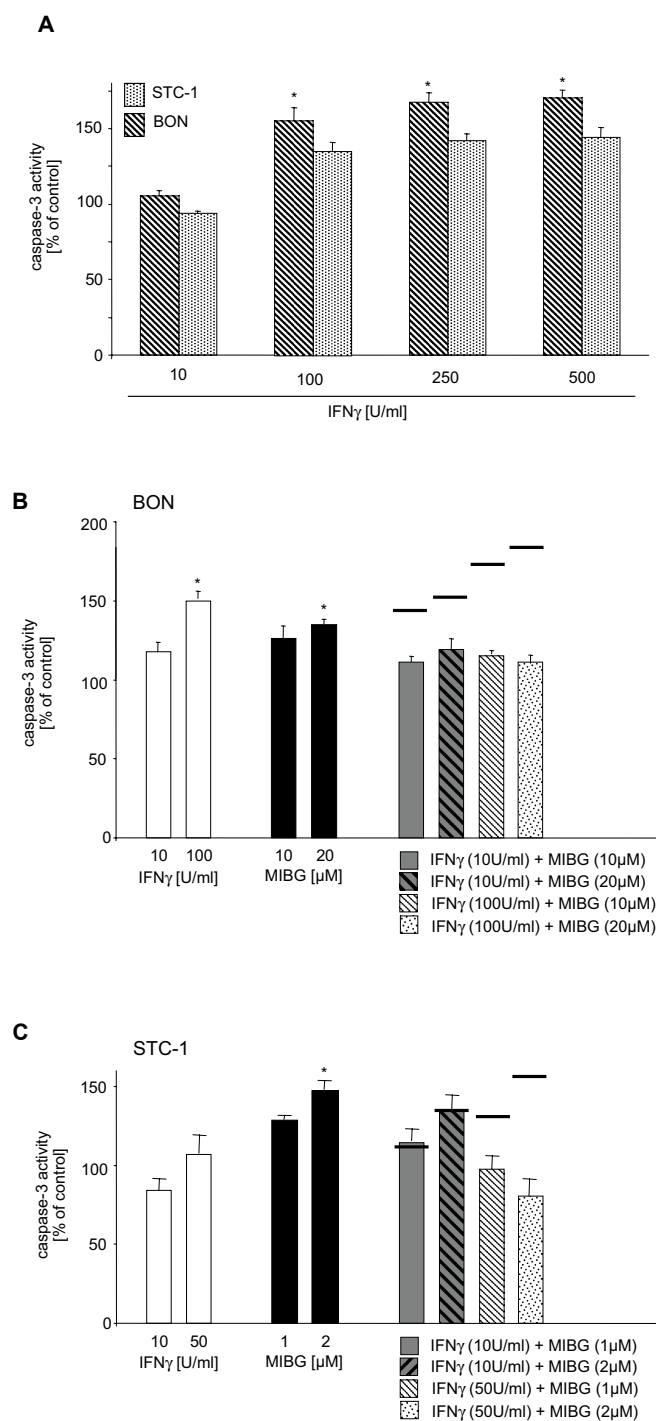
ml IFN $\gamma$  induced caspase-3 activation in both STC-1 and BON cells after 48 h of incubation (Fig. 6a). No significant increase in IFN $\gamma$ -induced caspase-3 activity was observed at concentrations higher than 100 U/ml or 250 U/ml IFN $\gamma$ . Unexpectedly, co-treatment with IFN $\gamma$  (10–100 U/ml) plus MIBG (1–2  $\mu$ M for STC-1 cells; 10–20  $\mu$ M for BON cells) led to subadditive caspase-3 activation in either BON or STC-1 cells (Fig 6b,6c).

#### IFN $\gamma$ -induced cytotoxicity

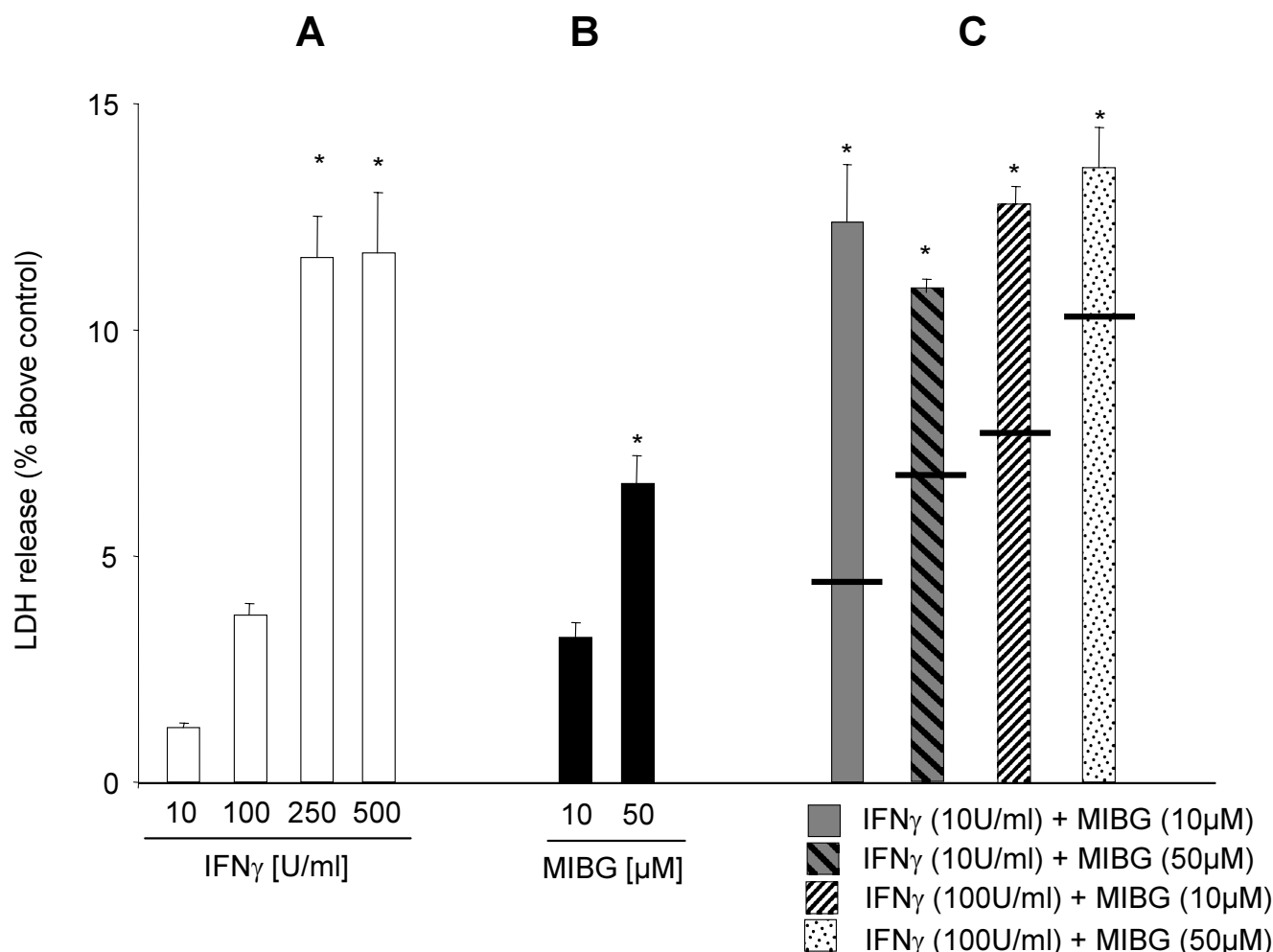
Cytotoxic loss of cellular membrane integrity was measured by LDH release assay. Incubating BON cells with 10–500 U/ml IFN $\gamma$  for 48 h resulted in a pronounced and

dose-dependent increase in LDH release (Fig. 7a). Additionally, we analyzed MIBG-induced cytotoxicity in BON cells. Treatment with MIBG for 48 h also dose-dependently increased the LDH-release of BON cells (Fig. 7b). The combination of IFN $\gamma$  (10–100 U/ml) and MIBG (10–50  $\mu$ M) resulted in a marked overadditive LDH release from BON cells (Fig. 7c). Compared to the calculated values of an assumed additive cytotoxicity (indicated as black bars in the columns of Fig. 7c), the actual LDH-release induced by 100 U/ml IFN $\gamma$  plus 50  $\mu$ M MIBG was about  $32 \pm 4\%$  higher than the calculated sum. The combination of the lowest doses of IFN $\gamma$  (10 U/ml) and MIBG (10  $\mu$ M) surmounted the calculated sum of each effect by  $188 \pm$



**Figure 6**

**Induction of apoptosis by IFN $\gamma$  and MIBG** **A:** IFN $\gamma$  induced apoptosis specific caspase-3 activity in BON and STC-1 cells after 48 h of incubation. **B:** MIBG-treatment (48 h) dose-dependently increased caspase-3 activity of BON cells. Interestingly, combination treatment with IFN $\gamma$  and MIBG resulted in sub-additive caspase-3 activation. Black bars indicate the calculated additive values of caspase-3 activation of either combination. **C:** MIBG-treatment (48 h) dose-dependently increased caspase-3 activity of STC-1 cells. Co-incubation with IFN $\gamma$  and MIBG led to sub-additive caspase-3 activation. Black bars indicate the calculated additive values of caspase-3. Data are given as means  $\pm$  SEM of 3 (STC-1) or 4 (BON) independent experiments. \*, statistical significance ( $p < 0.05$ ) compared with untreated controls.

**Figure 7**

**IFN $\gamma$  induced cytotoxicity.** Cytotoxicity of IFN $\gamma$  was evaluated by lactate dehydrogenase (LDH) release from IFN $\gamma$ -treated BON cells. **A:** After 48 h of incubation, IFN $\gamma$  induced a dose-dependent increase in LDH release. **B:** MIBG-treatment (48 h) also induced cytotoxicity in BON cells in a dose-dependent manner. **C:** Interestingly, co-treatment of BON cells with IFN $\gamma$  and MIBG resulted in an over-additive LDH release. Black bars indicate the calculated additive values of LDH release of either combination. Data are given as the relative increase in LDH release compared to untreated controls (means  $\pm$  SEM of 4 independent experiments). \*, statistical significance ( $p < 0.05$ ) compared with untreated controls.

12%. Thus, the cytotoxic effect of a "low-dose combination" of IFN $\gamma$  and MIBG was as pronounced as the one obtained by 500 U/ml IFN $\gamma$  alone.

## Discussion

Although excellent control of tumor-associated hypersecretion syndromes of neuroendocrine gastrointestinal tumors can be achieved by biotherapy with somatostatin analogs or IFN $\alpha$ , systemic treatment options to inhibit the growth and further spread of metastatic neuroendocrine tumors are still unsatisfactory. For instance, IFN $\alpha$  although excellently controlling hypersecretion in most

cases [2], has been shown to cause only a partial tumor remission in less than 20% of treated patients [1,7,26]. Novel treatment modalities are therefore needed.

Here, we report on the antineoplastic effects of IFN $\gamma$ , a cytokine which has already been shown to exert growth inhibitory effects *in vitro* in several non-neuroendocrine cancer models, including breast [27], hepatocellular [28], ovarian [29] and colorectal carcinoma [30,31] or glioblastoma [32]. Also *in vivo*, IFN $\gamma$  has been demonstrated to be beneficial as a first line therapeutic agent for ovarian cancer [33] or as a supportive drug for enhanced

chemoimmunotherapeutic regimes of metastatic melanoma [34]. Nevertheless, in other cancers (i.e., renal cell carcinoma or SCLC) IFN $\gamma$  did not show any appreciable beneficial antineoplastic action in man [35,36], arguing for a distinctive tumor species-dependent effect of the cytokine. As IFN $\gamma$ -mediated growth control of a particular tumor type does apparently not allow to predict similar effects in other tumor species, it was all the more important to investigate IFN $\gamma$ 's antineoplastic action in neuroendocrine gastrointestinal tumor cells. Here we provide evidence that IFN $\gamma$  potently inhibits growth of neuroendocrine gastrointestinal tumor cells. When IFN $\gamma$  was combined with MIBG, an even more pronounced antiproliferative effect was obtained.

As the growth pattern of NE gastrointestinal tumors exhibits a wide spectrum ranging from very slow to fast growing types [37], it was important to study IFN $\gamma$ -induced growth inhibition in NE tumor cell lines with different growth characteristics and of different origin. We chose two models of NE gastrointestinal tumor disease: First, human pancreatic carcinoid BON cells with a doubling time of 34 h, and second, NE tumor cells of the gut (STC-1 cells) with a doubling time of 54 h [38]. In either NE tumor model we observed growth inhibitory effects of IFN $\gamma$  with IC<sub>50</sub> values of about 100 U/ml and maximal growth inhibition by 75–90% at  $\geq 250$  U/ml IFN $\gamma$ . As these concentrations are within the therapeutically achievable range of IFN-plasma levels in humans [25], IFN $\gamma$  appears to be a promising agent for innovative growth inhibition of NE gastrointestinal tumor cells.

The signaling pathways mediating IFN $\gamma$ 's antineoplastic action differ between various tumor types and lead to distinct biological responses, such as G<sub>1</sub>-arrest [39], S-phase arrest [40,41], induction of apoptosis [24,28] and cytotoxicity [42].

To further characterize the antiproliferative action of IFN $\gamma$  in NE gastrointestinal tumor cells, we performed cell cycle analyses. Flow cytometry with isolated nuclei of IFN $\gamma$ -treated BON tumor cells, revealed a significant increase in the proportion of cells in the S-phase. Cell cycle arrest by IFN $\gamma$  in the S-phase had previously been described for other tumors such as glioblastomas [32], but other reports described a G<sub>0</sub>/G<sub>1</sub>-arrest in response to IFN $\gamma$  treatment [6,7,43]. The accumulation of NE gastrointestinal tumor cells in the S-phase of the cell cycle may be of great value for novel combination treatments with established antimetabolic chemotherapeutics such as 5-fluorouracil, which interfere with DNA-synthesis [44]. Thus, the S-phase arresting cytostatic drug irinotecan enhances the antiproliferative efficacy of 5-fluorouracil in colon cancer [45].

Moreover, we observed an induction of apoptosis in neuroendocrine gastrointestinal tumor cells by IFN $\gamma$ . Concerning the underlying mechanisms of IFN $\gamma$ -induced apoptosis both indirect mechanisms like an increase in TNF- $\alpha$  sensitivity [46], an upregulation of CD95 receptor expression [47] or a modulation of human leukocyte antigen molecules (HLA) [48], and direct mechanisms like the upregulation and activation of caspases have been shown [24,49]. In BON and STC-1 cells we found a dose-dependent induction of apoptosis via proapoptotic caspase-3 activation. This direct mechanism has been previously described by Detjen et al. [50] in neuroendocrine QGP-1 tumor cells. In OGP-1 cells IFN $\gamma$ -mediated apoptosis was found to require intermediate signaling steps such as induction of the interferon regulatory factor IRF-1 and subsequent activation of the initiator caspase-1, which accounted for the relatively long time to response of IFN $\gamma$ -induced apoptosis. Caspase-1-mediated activation of downstream caspases in response to IFN $\gamma$  has been reported for other cell models, like leukemia cells [51], vascular smooth muscle cells [52] and HeLa cells [53]. It is intriguing to speculate, that also in the BON and STC-1 tumor models IFN $\gamma$ -mediated apoptosis involved such intermediary signaling steps, since caspase-3 activation in BON and STC-1 cells was not detectable earlier than after 48 h of incubation with IFN $\gamma$ . In order to fully understand the signaling events of IFN $\gamma$ -induced apoptosis in neuroendocrine tumor cells further investigations are required.

Growth inhibition of NE tumor cells by IFN $\gamma$  also involves cytotoxicity. At least at high concentrations of IFN $\gamma$ , BON cells displayed a significant increase in LDH release. Interestingly, at the half-maximal growth inhibitory IFN $\gamma$  concentration of 100 U/ml neither a significant increase in cytotoxicity nor a significant S-phase arrest could be observed, and only caspase-3 activity was significantly increased. Thus, at lower concentrations IFN $\gamma$  appears to control growth of NE tumor cells mainly by apoptosis, whereas at higher concentrations other mechanisms are involved.

The second goal of our study was to explore the growth inhibitory effects of a combination treatment of IFN $\gamma$  and non-radiolabeled meta-iodobenzylguanidine (MIBG). The rationale for investigating this particular combination was first, that non-radiolabeled MIBG was a potent NE tumor-specific antineoplastic drug [11,38], and second, that IFN $\gamma$  enhanced the uptake of MIBG by increasing the expression of norepinephrine transporters in neuroblastoma cells [13]. Moreover, we recently showed that MIBG induced an overexpression of IFN $\gamma$ -receptors in NE gastrointestinal tumor cells [11]. These results suggested possible synergistic action of a combination treatment of IFN $\gamma$  and MIBG. Intriguingly, combining sub-IC<sub>50</sub>

concentrations of IFN $\gamma$  and MIBG led to growth inhibition of up to 95%. In STC-1 cells, the combination of IFN $\gamma$  and MIBG exceeded the maximal antiproliferative effect of IFN $\gamma$  alone by more than 20%. To achieve 90% growth inhibition of STC-1 cells by MIBG alone, concentrations beyond 25  $\mu$ M were needed. Thus, combining IFN $\gamma$  and MIBG allows to reduce the concentration of either agent to one fifth without losing excellent growth control. The same holds true for BON cells, which also displayed enhanced growth inhibition in response to sub-IC<sub>50</sub> concentrations of IFN $\gamma$  and MIBG. The potential of this novel combination treatment strategy will have to be evaluated in future clinical studies.

To further investigate underlying mechanisms of the proposed combination therapy, we evaluated IFN $\gamma$ -induced norepinephrine transporter expression, apoptosis, cell cycle regulation and cytotoxicity in BON cells. Our results indicate that an overexpression of norepinephrine transporters does not account for enhanced growth inhibition by IFN $\gamma$  plus MIBG. Although IFN $\gamma$  induced the expression of norepinephrine transporters (NET) in neuroendocrine gastrointestinal tumor cells, the IFN $\gamma$  concentrations required to achieve a marked increase in NET expression were far above the antiproliferative IC<sub>50</sub>-value of about 100 U/ml. Hence, our data indicate that an enhanced MIBG-uptake due to an IFN $\gamma$ -induced overexpression of NET cannot be achieved with IFN $\gamma$  concentrations, suitable for combination treatment.

Unexpectedly, the combination of MIBG and IFN $\gamma$  resulted in a sub-additive caspase-3 activation as compared to treatment with either drug alone. Furthermore, we found an abrogation of MIBG-induced G<sub>0</sub>/G<sub>1</sub>-phase arrest in BON cells that had been co-treated with S-phase arresting IFN $\gamma$ . Thus, MIBG and IFN $\gamma$  appear to modulate distinct signaling pathways to induce apoptosis and cell cycle arrest in NE tumor cells. Due to yet unknown interferences in the signaling events, the effects on apoptosis and cell cycle were less pronounced and even sub-additive. Thus, it will be of future interest to identify the signaling molecules involved in this interference to gain insight into the underlying regulatory processes at the molecular level. However, sub-additive cell cycle arrest has already been found for other drug combinations, like the combination of 5-fluorouracil and cisplatin in colon cancer. In contrast to our findings the sub-additive cell cycle arrest of 5-fluorouracil and cisplatin led to a reduced growth inhibition than with 5-fluorouracil alone [54].

Despite of the reduced apoptosis and cell cycle arrest the growth inhibition obtained by IFN $\gamma$  plus MIBG was additive in NE tumor cells. The most likely explanation of these conflicting results is a synergistic increase in cytotoxicity in response to IFN $\gamma$  plus MIBG. While almost no

cytotoxicity was seen for either drug alone, the combination of low doses of MIBG and IFN $\gamma$  led to a pronounced and significant synergistic increase in cytotoxicity. Concentrations as high as 500 U/ml were needed to achieve comparable cytotoxic effects by IFN $\gamma$  alone. The underlying mechanisms are yet unknown, but have to be studied in the future. Anyway, the very fact, that co-treatment of NE tumor cells with IFN $\gamma$  and MIBG exerts such a pronounced cytotoxic effect may be of potential clinical value: As the expression of MIBG-transporting norepinephrine transporters in extraneuronal locations is limited or low (e.g., heart, renal medulla, lung endothelial cells), the relative specific and exclusive NET (over)expression in neuroendocrine gastrointestinal tumor cells forms a basis for NE tumor-targeted treatment [55]. Thus, a low concentration of MIBG renders an ineffective concentration of IFN $\gamma$  cytotoxic in NE tumor cells. It can therefore be speculated that the combination of IFN $\gamma$  with MIBG may induce a NE tumor cell-specific killing with reduced side-effects in normal cells. Future clinical studies will have to clarify the actual effects *in vivo*.

## Conclusions

Our study provides evidence that IFN $\gamma$  induces both cell cycle arrest and apoptosis in NE gastrointestinal tumor cells. Moreover, IFN $\gamma$  can be effectively combined with non-radiolabeled MIBG in order to achieve excellent growth control at low concentrations of either drug. Thus, IFN $\gamma$  represents a promising novel drug to be tested in patients with metastatic NE gastrointestinal tumor disease.

## List of abbreviations

IFN $\gamma$ , interferon gamma

IRF, interferon regulatory factor

MW, molecular weight

MIBG, meta-iodobenzylguanidine

PBS, phosphate-buffered NaCl solution

RT-PCR, reverse transcriptase-polymerase chain reaction

STAT, signal transducers and activators of transcription

## Competing interest

None declared.

## Authors' contributions

MH : writing, study design, experimental design, investigation of growth inhibition and apoptosis; APS: cell cycle analysis by flow cytometry; AH: cytotoxicity studies; G A-H: RT-PCR, interpretation of the data; HS: study design,

writing. All authors read and approved the final manuscript.

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